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(54) Title: BIOCHEMICAL REAGENT

(57) Abstract

A biochemical reagent comprises an oligosaccharide, preferably one which has been liberated from an immunogenic glycoprotein or proteoglycan, which is immobilised on a carrier via an intermediate spacer molecule such as a lipid. The lipid molecule should preferably have at least two long lipid tails so that the oligosaccharide is held in spaced relationship to the carrier where it exhibits antibody-binding ability which is almost indistinguishable from that of the original glycoprotein or proteoglycan. The reagent has its application in biochemical treating of oligosaccharides and systems which bind to them.

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BIOCHEMICAL REAGENT

This invention relates to a biochemical reagent, its preparation and its uses. More particularly, the reagent of this invention is an immobilised oligosaccharide and has its principal application in the biochemical investigation of oligosaccharides which occur in combination with proteins, as glycoproteins and proteoglycans, and which may be immunologically active.

Production of antibodies by the body is triggered by an encounter between a foreign molecule (an "antigen") and cells of the adaptive immune system. The biochemical mechanisms of antibody production are complex and have not been fully elucidated. What is known is that antigens trigger the immune system to produce antigen-specific antibodies which on subsequent encounters will bind to the antigen resulting in its inactivation. Regions on the antigen to which antibodies bind are known as "antigenic determinants" or, synonymously, "epitopes". In the interests of simplicity, the term "antigenicity" is used

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herein and in the claims to indicate the ability of a
molecule to bind to an antibody.

It is known that when antibodies bind to proteinaceous
antigens such as glycoproteins and proteoglycans, the
epitope to which the antibody is directed may be a sequence
of amino acids on the protein or a sequence of sugar units
on one of the glyco group oligosaccharide chains.
Investigation of the antigenicity of the oligosaccharides is
of significant biochemical and medical interest.

Oligosaccharides, when isolated as such from
immunogenic glcoproteins and proteoglycans, do not bind to
their known antibodies in vitro; for example, their simple
solutions do not bind to antibodies. The probable reason
for the absence of antibody-binding ability is that epitopes
are only recognised by antibodies when they are presented to
the antibodies in specific orientation and concentration.

In Analytical Biochemistry, 139, 168-177, Raja, R.H.
et.al. describe a method of bonding oligosaccharides,
obtained by enzyme degradation of hyaluronic acid, to
immobilising supports to give, for example, affinity
chromatography media and polyacrylamide cell culture
surfaces. Hyaluronic acid is a polysaccharide, occurring
in connective tissue, with a molecular weight of several
million and composed of repeating units of
N-acetylglucosamine and glucuronic acid. Partial digestion
of hyaluronic acid with the enzyme hyaluronidase, under the
conditions descibed by Raja,

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produces a mixture of fragments of which about fifty percent
have an average size of from six to eight disaccharide units
(i.e. from twelve to sixteen monosaccharide units). This
oligosaccharide mixture is bonded to the immobilising
support by a method which involves the following sequential
steps: (a) reduction of the oligosaccharide with sodium
borohydride, which results in cleavage of the terminal sugar
ring and formation of a methylol group at the site of the
ring opening, (b) selective oxidation of the methylol group
with sodium periodate to form an aldehyde group, (c)
coupling of the aldehydo-oligosaccharide to an alpha, omega
alkyldiamine such as hexane diamine in the presence of
sodium cyanoborohydride resulting in a derivative which has
the oligosaccharide residue at one end of a polymethylene
chain and an active amino group at the remote terminus
(omega postion); and, (d) bonding of the derivatized
oligosaccharide, via the omega amino group, to a
chromatography support, such as Sepharose (Trade Mark),
under the action of cyanogen bromide or, alternatively, to a
polyacrylamide cell culture surface under the action of
sodium cyanoborohydride.

It is known that reducing disaccharides (i.e. those
having active aldehyde groups) may be coupled to proteins or
to aminoethyl polyacrylamide gels by selective reduction of
a Schiff base, formed between the reducing (aldehyde) group
of the disaccharide and the amino group of the protein or
the aminoethyl gel using sodium cyanoborohydride [Gray,
G.R., (1974), Arch. Biochem. Biophys.; 163, 426-428]. It is

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the reduction method of Gray which is utilised by Wood and
Kabat to produce conjugates of isomaltosyl oligosaccharides
with lipids [J. Exp. Med., 154, 432-449, (1981)].   In Wood
and   Kabat's   immunological   study   of   isomaltosyl
oligosaccharides, the oligosaccharides are conjugated to
stearylamine (octadecylamine).   The stearyl isomaltosyl
oligosaccharide conjugate was found to trigger production of
immunoglobulins (i.e. the conjugate was immunogenic) when
injected into test rabbits, provided it was first emulsified
with complete Freund's adjuvant (CFA) in which, it is said,
the conjugate forms miscelles.   The conjugate was also
found to induce antibody production in rabbits when it was
incorporated into liposomes.   It is reported that the
larger oligosaccharides are rather more active than the
shorter, the suggestion being that the shorter chains do not
protrude sufficiently from the liposome surface to be active.

An object of the present invention is to enable the
analysis of the interaction of oligosaccharides and
molecules to which they bind by providing materials and
methods directed to this end.

According to the present invention there is provided a
biochemical reagent comprising an antigenic conjugate
between an oligosaccharide and an immobilising carrier, said
oligosaccharide being bound in spaced relationship to the
carrier by means of an interposed non-antigenic spacer
molecule whereby the oligosaccharide is presented in
antigenically active steric configuration for binding on
encountering its antibody.

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The term "antibody" is used herein is to be construed as including all carbohydrate-binding moieties.

The invention also provides a method for the screening of antibodies which comprises contacting a solution containing an antibody with the immobilised antigenically active conjugate aforesaid and thereafter detecting the occurrence (or non-occurrence) of binding.

The spacer molecule is preferably a lipid molecule and, more preferably, a glyceride or phosphatide which possesses at least two hydrophobic polyalkylene chains. The combination of the oligosaccharide and the lipid is hereafter referred to as a "neoglycolipid".
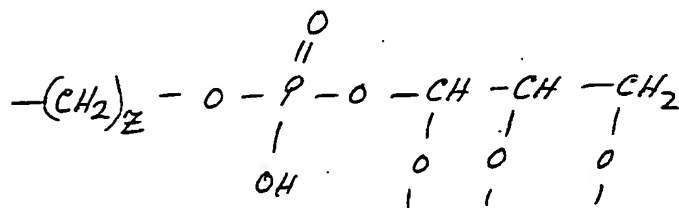
The spacer molecule may be a long chain fatty acid of from 5 to 50, preferably 5 to 30 and more preferably 10 to 25, carbon atom length, an example being stearylamine and similar amino derivatives of lauric, myristic and palmitic acids.   Simple amino hydrocarbons may also be employed. The amino group is necessary for chemical bonding to the oligosaccharide to form the antigenic conjugate.

Neoglycolipids   may   be   formed   by   reacting   an oligosaccharide having at least one aldehyde group with an amine having one or more hydrophobic groups.

Preferably the neoglycolipid has the following formula:

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where R^1 represents a carbohydrate group,

    R^2 represents an alkyl, or alkoyl group,

    R^3 represents an alkyl, or alkoyl group,

    R^4 represents an alkyl, or alkoyl group, or hydrogen

R^2, R^3 and R^4 being the same or different groups, z is zero or 1,2 or 3 and X represents a linking group which may be selected from:

$$-(CH_2)_z-O-\overset{\overset{O}{\|}}{\underset{\underset{OH}{|}}{P}}-O-\overset{\underset{\underset{|}{O}}{|}}{CH}-\overset{\underset{\underset{|}{O}}{|}}{CH_2}$$

$$-(CH_2)_z-O-\overset{\overset{O}{\|}}{\underset{\underset{OH}{|}}{P}}-O-\overset{\underset{\underset{|}{O}}{|}}{CH}-\overset{\underset{\underset{|}{O}}{|}}{CH}-\overset{\underset{\underset{|}{O}}{|}}{CH_2}$$
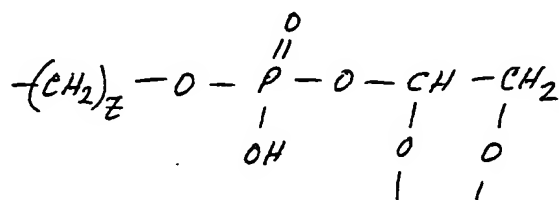
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Neoglycolipids of particular interest, have the general formula:

$$R^1 - NH \underline{\quad\quad} CH_2 - CH_2 - O - \overset{\overset{\displaystyle O}{\|}}{\underset{\underset{\displaystyle OH}{|}}{P}} - O - CH_2 - \underset{\underset{\underset{\underset{\underset{\displaystyle CH_3}{|}}{(CH_2)_n}}{|}}{\underset{\displaystyle CO}{|}}}{CH} \underline{\quad\quad} \underset{\underset{\underset{\underset{\underset{\displaystyle CH_3}{|}}{(CH_2)_m}}{|}}{\underset{\displaystyle CO}{|}}}{CH_2}$$
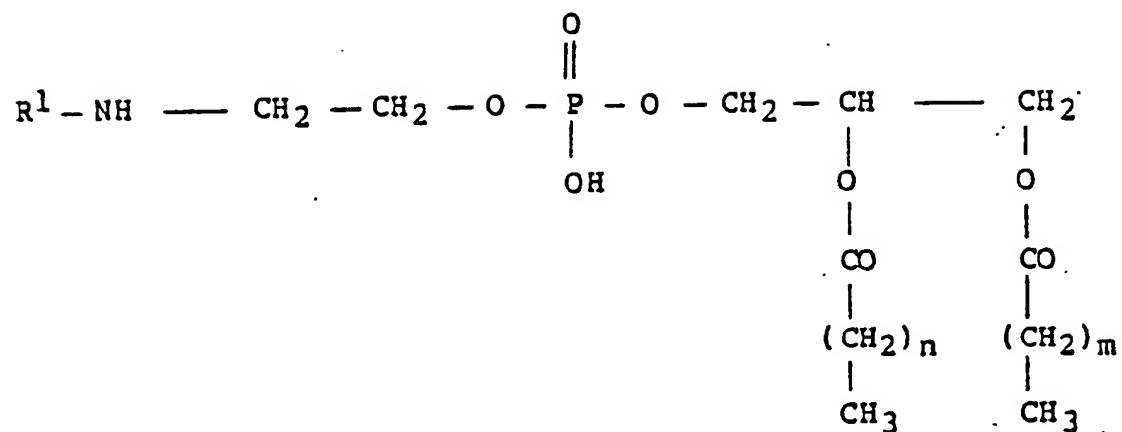
where the integers n, and m have a value within the range 5 to 50, preferably 5 to 30, and desirably 10 to 25.

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The carbohydrate group R^1 may be derived from a monosaccharide, oligosaccharide, or even a polysaccharide which contains an aldehyde group. A carbohydrate group R^1, which does not in itself contain the necessary aldehyde group, may be subjected to oxidation, for example using periodate oxidation. Of particular biochemical interest are carboyhdrate groups which can be isolated from glycoproteins and proteoglycans by a reduction treatment resulting in a reduced oligosaccharide which contains a methylol group, in which case, to form the necessary aldehyde they may be subjected to a periodate oxidation treatment as described by Raja et al, in Anal. Biochem, 1984, Vol 139, pages 168-177.

The most preferred spacer compounds are represented by phosphatidyl ethanolamine dipalmitoyl (PPEADP) and its analogues, L-alpha- phosphatidyl-L-serine, L-alpha-phosphatidyl- ethanolamine (PPEA) dilauroyl, PPEA dimyristoyl, PPEA distearoyl, PPEA beta-oleoyl-gamma-palmitoyl.

The immobilising carrier may be a plate of plastics material such as polyvinyl chloride, a chromatographic support material such as silica gel or a liposome.

In preferred embodiments of the invention the oligosaccharide is obtained from a naturally-occurring glycoprotein or proteoglycan.

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Oligosaccharides may be bound to proteins via an oxygen atom (O-linked) or via a nitrogen atom (N-linked). Two different approaches are necessary for the liberation of the oligosaccharides from their bound protein by chemical means. Treatment with alkaline sodium borohydride will break the O-link and simultaneously open the terminal saccharide ring with formation of a methylol group at the ring cleavage site (Iyer & Carlson, Arch. Biochem. Biophys., 1971, Vol 142, pages 101 to 105).. Similarly, N-linked oligosaccharides may be obtained and purified in the reduced state (Fukuda et al J Biochem. 1976, Vol 80 pages 1223-1232 and Takasaki et al. Methods Enzymol 1982, vol 83, pages 263-268) and suffer from the limitation described above in that, being fully reduced they are not reactive per se and require oxidation to introduce an aldehyde group. Alternatively, the oligosaccharides may be liberated from their bound protein by enzyme action.

Reference is made to Fig.1 of the accompanying drawings, which shows a reaction scheme for the liberation of O- and N-linked oligosaccharides from glycoproteins and proteoglycans and their reaction with lipids to form neoglycolipids. The pyranose ring structure shown in Fig.1 is shown as N-acetylglucosamine for the purpose of illustration only and is to be construed as representative of a terminal saccharide ring of any oligosaccharide, the remainder of the oligosaccharide molecule being represented by the symbol [Oligo].

An N-linked protein bound oligosaccharide (structure

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I) is subjected to hydrazinolysis which breaks the linkage
between the saccharide and its bound protein (represented by
[Protein]) with formation of an hyrdazido group at the site
of the original saccharide/ protein link (structure II).

Acetylation with acetic anhydride and
neutralisation converts the hydrazido group to a hydroxyl
group and re-N-acetylates the amino group (structure III).

Treatment of the hydroxy compound shown in structure
III with sodium borohydride cleaves the terminal saccharide
ring structure with formation of a methylol group ($-CH_2OH$)
at the site of ring cleavage (structure IV).

An oligosaccharide bound to a protein via an O-link
(structure V) is treated with sodium hydroxide/sodium
borohydride which cleaves the terminal saccharide ring with
formation of a methylol group at the cleavage site
(structure IV).

The methylol compound shown as structure IV is fully
reduced and is unreactive with amines. Selective oxidation
of the methylol group using sodium periodate under strictly
controlled conditions, of which details will be given
hereinafter, results in the reducing (aldehydo)
oligosaccharide shown as structure VI.

The reducing oligosaccharide (structure VI) is reacted
with an lipid amine such as is represented by the
phosphatide indicated in Fig.1, in the presence of sodium
cyanoborohydride to give the neoglycolipid (structure

VII).   The R groups of the phosphatide are as hereinbefore
described..

It is possible to react the sugar alcohol, shown as
structure III, directly with the cyanoborohydride and lipid
to obtain the neoglycolipid VII but this route (indicated by
the broken line in Fig.1), although removing several steps
of the multi-step reaction scheme, results is a product
which may contain isomeric forms of the neoglycolipid and
its utility is thus reduced.

Therefore, according to the present invention, there
is provided a method for preparing a biochemical reagent
comprising liberating an oligosaccharide moiety of an
immunogenic glycoprotein or a proteoglycan under reduction
conditions to form thereby a reduced oligosaccharide,
selectively oxidising the reduced oligosaccharide with
periodic acid or a salt thereof to form an aldehydo-
oligosaccharide,   and   conjugating   the   aldehydo-
oligosaccharide with an immobilising carrier via a spacer
molecule containing an amino group.

While not wishing to be bound by this particular
theory, the following is given by way of explanation of the
invention.   It is known that when antibodies are generated
<u>in vivo</u> in response to glycoprotein and proteoglycan
immunogens they often display specificity for epitopes on
the oligosaccharides which make up the glyco group.   The
binding of antibodies is a stereospecific mechanism which
depends on the ability of an antibody to recognise a
complementary

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molecular configuration on an epitope.   Both of the short
arms (Fab) of the antibody seek epitopes and bind thereto
and so it is evident that correct three-dimensional
presentation of the epitopes with respect to orientation and
spacing between pairs of epitopes, and with respect to the
physical separation of each epitope from the carrier matrix
is essential to ensure efficient and stable binding of the
antibody via both of its arms.   It is probable, then, that
regeneration of the antigenicity of oligosaccharides
released from glycoproteins and proteoglycans requires that
the epitopes be presented in a stereospecific configuration
and, it may be postulated, in a manner which allows some
molecular flexibility to enable the antibody arms to locate
two epitopes and draw them into the required lateral
displacement.   Oligosaccharides appear to lose their
antigenicity when released from glycoproteins and
proteoglycans, i.e. they are in the free state, nor are
short chain oligosaccharides rendered antigenic by mere
direct binding to an immobilising carrier.   When bound to a
carrier via short chain spacer molecules the antigenicity of
short chain oligosaccharides is present but may be weak.
Long   chain   oligosaccharides   do   display   some   weak
antigenicity when bound to a carrier via short chain spacer
molecules.   There does, therefore, appear to be some
relationship between antigenicity and the spacing between
the epitope and the surface of the carrier.   The
antigenicity of the conjugate of the invention may be
optimised   by

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experimentation with spacer molecules of various chain lengths. However, in practical terms, the oligosaccharides of biochemical interest have normally no more than about seven or eight sugar units and for molecules of this size spacers with twelve to eighteen carbon chains allow the epitope of the sugars to be presented antigenically. The second feature which is thought to participate in the binding mechanism is the lateral spacing between the epitopes. It is well known that the hydrophobic tails of lipid molecules tend to aggregate in solution . It has been found that antigenicity can be optimised by using lipid spacer molecules which have at least two hydrophobic lipid tails. This is believed to induce localised clustering of the tails on the carrier which should affect the lateral spacing of the epitopes at the remote end of the lipid/oligosaccharide conjugate. This postulate is supported by the observation that double-tailed conjugates have greater antigenicity than single-tailed conjugates of the same oligosaccharide, presumably because of an enhanced clustering effect induced by the multiple hydrophobic tails.

This invention also relates to a method for evaluating the antigenicities and/or receptor functions of oligosaccharides released from glycoproteins and proteoglycans.

There is an increasing awareness of the importance of carbohydrate chains of glycoproteins and proteoglycans of animal cells as onco-developmental antigens recognised by monoclonal antibodies, and as receptors for infective agents

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as well as endogenous ligands. Consequently there is a need
for a micromethod for evaluating the antigenicities and/or
receptor functions of oligosaccharides released from the
peptide moieties of glycoproteins or proteoglycans by the
methods described above. Although several hundreds of
milligrams of immunogenic proteins may be available as
starting material, the amount of individual purified
oligosaccharides, released from the glycoproteins, is very
small.

The method of this invention may be performed using
minute amounts of oligosaccharides by converting them into
neoglycolipids prior to assay.

Therefore, according to one aspect of the present
invention there is provided a micromethod for evaluating the
antigenicities and/or receptor functions of an
oligosaccharide comprising,

(a)   reacting a reducing oligosaccharide with an amine
        spacer molecule having the formula

$$H_2N - X \begin{array}{c} \diagup R2 \\ - R3 \\ \diagdown R4 \end{array}$$
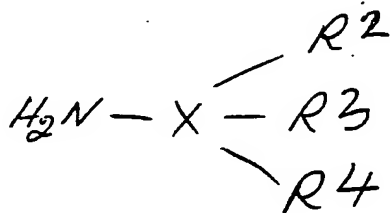
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where R^1 represents an alkyl or alkoyl group, R^2 represents an alkyl or alkoyl group or hydrogen, R^3 represents an alkyl or alkoyl group or hydrogen, R^1, R^2 and R^3 being the same or different, and X represents a linking group to form a neoglycolipid,

(b)   conjugating the neoglycolipid on to an immobilising carrier; and,

(c)   contacting the conjugate with an antibody or other carbohydrate . binding system and observing the occurrence (or non-occurrence) of binding of the conjugate.

Preferably the said micromethod includes the step of liberating protein-bound oligosaccharide from the protein and selectively oxidising the oligosaccharide with a salt of periodic acid under controlled conditions to form a reactive reducing oligosaccharide.

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Particularly preferred spacers molecules are the derivatives
where the R groups are alkoyl groups derived from the fatty
acids lauric, myristic, palmitic and stearic acids.   Longer
or shorter chain lengths may be appropriate in particular
circumstances but chains of 12 to 18 carbon atoms are
appropriate for most purposes, being a compromise between
the shorter chains which may only produce antigenicity with
longer chain oligosaccharides, and longer chain spacer
molecules which impart no significant enhancement of the
antigenicity and which are not readily available.   As will
be later illustrated in the Examples, it is apparent that
there is a correlation between the chain length of the
spacer and the induction of antigenicity.

The extent of binding of the conjugated
oligosaccharide of the invention with an antibody may be
determined by any of the well known methods.   For example,
radioactively labelled antibodies or carbohydrate receptor
system may be used and the amount bound to the
oligosaccharide detected by means of the radioactive label.
Where unlabelled antibodies are bound, radio-labelled
secondary antibodies specific to the relevant class of
unlabelled antibody (e.g. anti-mouse IgG antibody) may be
added, and after incubation the degree of radioactivity
measured.

Especially when the neoglycolipids are derived from

PPEADP, their antigenic activity is virtually indistinguishable from its activity on the glycoprotein or proteoglycan from which the oligosaccharide is isolated. Thus the conjugate of the invention enables the oligosaccharide moiety to:

(a)   be recognised by antibodies or carbohydrate binding systems, or

(b)   compete with other molecules having antibody or carbohydrate binding activity.

Thus the test method may be used for characterising physiological status, or for the typing of tissues, cells or infective agents.

An alternative assay enables the identification of oligosaccharides by contacting same with an immobilised antibody so that oligosaccharides containing an epitope recognised by the antibody will be bound to the immobilised antibody thereby confirming its identity.

In general, the assay method of the invention enables the study of the interaction between specific oligosaccharide structures and antibodies. It therefore provides a valuable tool for:

1.   basic research, for characterising antibodies;

2.   basic research, for characterising oligosaccharide structures, that is, using specific antibodies as structural reagents;

3.   diagnosis, for measurement of quantitative or

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qualitative changes in oligosaccharides or antibodies;
4.   purification of antibodies; and
5.   therapy.

The following are specific examples of the assay procedures which may be performed using the reagent of this invention:

I   Determination of Antibody binding characteristics

Oligosaccharides are immobilised in a conjugate of the invention and the binding of antibodies thereto are measured. The oligosaccharides may be homogeneous or derived from a particular immunogenic glycoprotein or proteoglycan, cell fraction, cell, tissue or body fluid. For example, the assay method of the invention may in particular be used to diagnose the nature or status of a disease in a living organism. This may be achieved for example when antibodies with specific oligosaccharide-binding characteristics are produced in the organism as a result of a disease state, and are hence diagnostic for that state. In this procedure, antibodies would be isolated from, for example, plasma, and their specificity determined by performing binding assays to conjugates of the invention which contain a range of oligosaccharides.

II   Determination   of   the   characteristics   of
        oligosaccharides

Using the procedure described in paragraph I, specific

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antibody/conjugate complexes are selected. Then a test preparation of conjugate, in which the carrier is a liposome and the identity of the oligosaccharide is unknown, is allowed to compete with plate-bonded conjugate for the bound antibody. Removal of the antibody by the test antigen indicates the similarity between the structures of plate-bound and liposome-bound oligosaccharides. This procedure allows characterisation of oligosaccharides and typing of cells according to their oligosaccharide-binding ability.

III   <u>Determination of the characteristics of cells and microorganisms</u>

A conjugate containing a specific oligosaccharide is bound to a PVC plate and cells or microorganisms are tested directly for their ability to bind to the plate. This method enables the typing of cells or strains of microorganisms.

IV   <u>Isolation of Antibodies</u>

By passing a solution of antibodies over a conjugate of this invention containing an oligosaccharide of interest, the antibody to the oligosaccharide will bind, allowing the remainder to be washed away and the linked antibody thereafter released in purified form.

V   <u>Effector Function</u>

A conjugate of the invention may be administered <u>in</u>

vivo to produce an immune response, for example, as an antagonist, the conjugate may compete with the oligosaccharides on the surface of infective microorganisms for binding sites on receptor cells, and vice versa, thus preventing infection. Also, as an agonist or antagonist to modify the function of glycoprotein hormones or glycoprotein receptors whose functions are mediated by carbohydrate structures.

The invention will now be described, by way of illustration in the following Examples.

EXAMPLE 1

Thin Layer Chromatography

The following experiments were conducted to illustrate the binding of the neoglycolipid to silica gel and to elucidate the relationship between the chain lengths of the spacer molecule and the oligosaccharide. At the same time optimum conditions for the binding were investigated.

Eight different amines as spacer molecules were investigated in three experiments. Oligosaccharides were chromatographed on high performance silica gel TLC plates using standard methods and the plates were then oxidised with periodate followed by reaction with the amine and sodium cyanoborohydride to bind the oligosaccharide fractions to the plate.

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The method employed for the periodate oxidation was as follows:

1.   From 3 to 5 micrograms of the oligosaccharide are applied to appropriate lanes of a HP-TLC plate.

2.   The chromatogram is developed in an appropriate solvent system, for example butan-1-ol: acetone: water 6:5:4 by volume, and the plate then dried and cut into lanes.

3.   A solution of 0.02% of sodium periodate in a mixture of 40mM imidazole hydrochloride (pH 6.5):chloroform: methanol 1:5:5 by volume is prepared, cooled to $4^{\circ}C$ and stored in the dark.

4.   Each lane of the TLC is immersed in the periodate solution for ten seconds. The oragnic solvent is allowed to evaporate in air for a few seconds.

5.   The strips are immediately placed in a humid box at 4° C in the dark and maintained therein for 45 minutes.

6.   Each lane is then immersed in a 0.5% w/v solution of the coupling amine (usually PPEADP) in chloroform: methanol 1:1 v/v for ten seconds

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7.   The lanes are then placed in an air oven at 60°C for
      a period of one hour.

8.   Each lane is then immersed into a 0.2% w/v solution of
      sodium cyanoborohydride in chloroform: methanol 1:1
      for ten seconds.

9.   The lanes are replaced in the 60°C air oven for a
      further two hours.

It was the protocol described above which was employed
in all the chromatograms discussed in this Example, various
amines being employed to determine the extent of the binding
and the antigenicities of the various amines as spacer
moleculed for the conjugates.

In addition, a further test was undertaken to
investigate the influence of the periodate oxidation step on
the binding properties of the conjugate.   Dextran
hydolysate was used as the test oligosaccharide which was
oxidised with periodate by the protocol described, the
concentration of the periodate was varied to attempt to
ascertain an optimum value.   The chromatogram was stained
with orcinol and then washed.   The density of the stained
bands was assessed.

As a result of this test it was found that the lower
limit for periodate concentration was 0.005% and the upper
0.05% with the optimum at 0.02% which was confirmed in the
practical use of the invention in TLC binding assays.

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The results are tabulated below in Tables I to III.
In the experiments leading to the results shown in Tables I
and II, the conjugated oligosaccharides were exposed to
anti-Ma antibody or anti-5D4 antibody followed by a
radiolabelled second antibody then washed and
autoradiographed. The results presented in Table III were
produced by direct orcinol staining of the oligosaccharide
moiety of the conjugate to determine the level of binding to
the TLC plate. An assessment of the density of the bands
was made (on a scale of 0 to 1, the maximum being indicated
by 1), giving an indication of the binding efficiency.

Table I shows the effect of using various spacer
amines on the antigenicities of a trisaccharide. Table II
gives the results using a dodeca-saccharide, and, the data
in Table III demonstrates the level of binding to TLC plates
of a mixture of oligosaccharides derived from dextran
hydrolysate, as a function of the type of spacer molecule.

TABLE I

Immunostaining of trisaccharide v Ma antibody

AMINE	DENSITY
PPEAD myristoyl	1.0
PPEAD stearoyl	1.0
PPEADP	1.0
PPEA oleoyl palmitoyl	0.5
PPEAD lauroyl	0.1
Sphingosine	not detectable
Stearylamine	not detectable
PPS	not detectable

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TABLE II

Immunostaining of J1 Dodecasaccharide v 5D4 Antibody

AMINE	DENSITY
PPEAD myristoyl	1.0
PPEADP	0.9
PPEAD stearoyl	0.8
PPEA oleoyl palmitoyl	0.5
PPS	0.5
Stearylamine	0.2
PPEAD lauroyl	0.2
Sphingosine	trace

TABLE III

REDUCED DEXTRAN HYDROLYSATE with ORCINOL STAIN

AMINE	DENSITY
PPEAD lauroyl	1.0
Stearylamine	0.8
PPEAD myristoyl	0.8
PPEAD oleoyl palmitoyl	0.8
PPS	0.5
PPEAD stearoyl	0.5
PPEADP	0.5
Sphingosine	0.2

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The various amines used in generating the data given in Tables 1 to 3 are characterised as follows:

AMINE	M.W.	NOTES
L-alpha-phosphatidyl-L-serine (PPS)	313	no lipid tail
D-sphingosine	300	single C_{17} tail
L-alpha-phosphatidylethanolamine (PPEA) dilauroyl	580	two C_{13} tails
PPEA dimyristoyl	636	two C_{15} tails
PPEA dipalmitoyl	692	two C_{17} tails
PPEA distearoyl	748	two C_{19} tails
PPEA-beta-oleoyl-gamma-palmitoyl	718	one C_{17} tail, and C_{19} tail with double bond

By comparing the results given in Tables 1 to 3 above, the following conclusions can be drawn:

(a)   The antigenicity of a short chain oligosaccharide (tri-
saccharide: Table 1) bound with short chain spacer amines such as PPS, is low;

(b)   The antigenicity of a short chain saccharide, Table 1, bound with spacer amines with double lipid tails, such as PPEADP myristoyl and stearoyl, is high;

(c)   The antigenicity of a long chain oligosaccharide (dodecasaccharide, Table 2) is relatively strong even with short spacer amines such as PPS;

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(d)   The antigenicity of a long chain oligosaccharide
        (Table 2), bound with spacer amines with single lipid
        tails, such as sphingosine, is weak;

(e)   The strength of binding of the neoglycolipid to the
        silica gel, as opposed to the antigenicity of the
        conjugate, (Table 3) is not of the same order as
        indicated for the antigenicity. For example, whereas
        PPEAD lauroyl binds the oligosaccharide more strongly
        than, for example, PPEADP (Table 3), the antigenicity
        of the PPEAD lauroyl complex is much lower for both
        tri- and dodeca-saccharides (Tables 1 and 2).

(f)   The binding strength of neoglycolipids with single
        tails may be high (stearylamine in Table 3) but the
        antigenicity is low in comparison with double tailed
        molecules (Tables 1 and 2).

EXAMPLE 2

Materials

(a) Oligosaccharides

The oligosaccharides used were: lactose, maltose,
tetrasaccharide N_1, obtained from human meconium
glycoproteins [Hounsell E.F. et al , Eur. J. Biochem 148,
(1985) 367-377] and, chemically synthesised disaccharide
N-acetyllactosamine, trisaccharide (O_4) and
pentasaccharide (O_8) [Auge, C., David,S., & Veyrieres, A.;
(1979), Nouveau J. de Chimie, 3, 491-497], and chemically
synthesised 3-fucosyl-N-acetyllactosamine (3F). The
synthesised saccharides were reduced with sodium

borohydride.   Lacto-N-fucopentaitol III (LNFP III alditol)
was derived from a fraction of human milk pentasaccharides.

(b) Monoclonal Antibodies:

(i)   human   serum,   Ma,   containing   a   monoclonal   anti-I
       antibody, at a dilution of 1:1000, and,
(ii)   mouse hybridoma antibody, anti-SSEA-1, at a dilution
       of 1:100.

(c) Lectin

The plant lectin Ricinus communis agglutinin, which contains
a carbohydrate binding system.

Controlled Oxidation

The   reduced   oligosaccharides   (10   micrograms)   were
oxidised   with   sodium   periodate   (4   equivalents)   in   100
microlitres   of   40mM   imidazole   hydrochloride   (pH   6.5)   at
$0^{o}C$ for   one   hour.   The   resultant   solutions   were   used
immediately   for   combination   with   L-alpha-phosphatidyl-
ethanolamine dipalmitoyl (PPEADP).

Combination with PPEADP

Lactose   and   maltose   (10   micrograms)   or   50   microlitres
of   the   prepared   solutions   of   the   periodate   oxidised

oligosaccharides were added to 150 microlitres of a
chloroform/methanol (1:2 v/v) solution containing 100
micrograms of PPEADP. The solutions were allowed to react
at 50°C for two hours. Sodium cyanoborohydride (50
micrograms in 10 microlitres of methanol) were then added
and the solutions again maintained at 50°C for a further
116 hours. Thereafter the solvents were evaporated from
the solutions under nitrogen and the residues redissolved in
40% v/v aqueous methanol. Dilutions of 10, 1.0, and 0.1
micrograms of carbohydrate per millilitre in 40% v/v aqueous
methanol were prepared for PVC plate-binding assays.

Binding to Carrier Matrices

(a) Binding to Plates

Aliquots of 50 microlitres of the solutions of
neoglycolipids, prepared as described by combination of the
disaccharides, oligosaccharides with PPEADP, were added to
the wells of round-bottomed flexible PVC microtitre
plates. The plates were held at 4°C for 90 minutes and
then the wells were washed five times with phosphate
buffered saline (PBS) of pH 7.4, and treated with a 3% w/v
solution of bovine serum albumin (BSA) in PBS at 37°C for
one hour. The wells were then washed five times with PBS.

(b) Chromatographic Carrier

For chromatogram binding assays , the reduced
oligosaccharide O_4 and lactose (3 micrograms per lane)

were chromatographed on HP-TLC plates (aluminium sheets with silica gel 60 from E. Merck) using a solvent system butan-1-ol/acetone/water 6:5:4 v/v. The chromatograms were dipped into a chloroform/methanol/40mM imidazole hydrochloride solution (pH 6.5) containing 0.02% w/v of sodium periodate and left at $4^{\circ}C$ for for 45 minutes and then dipped into a 1:1 v/v chloroform/methanol solution of 0.5% w/v PPEADP. They were then maintained at $60^{\circ}C$ for one hour and then dipped into a solution of 0.2% w/v of sodium cyanoborohydride in chloroform/methanol 1:1 v/v and incubated at $60^{\circ}C$ for a further two hours.

Chomatogram Binding Assay

The chromatograms were then either stained with orcinol stain or were overlaid with 5% BSA in PBS for two hours at room temperature and immunostained with anti-I(Ma) 1:100 dilution or control serum (Ns) followed by ^{125}I-labelled anti-human IgM, as described in Uemura, K. et.al. (1983) Biosci. Reps., 3, 577-588, and autoradiographed using Singul-X RP (Trade Mark) X-ray films.

Plate-binding Immunoassay

To the wells of the prepared plates, 50 microlitres of antibody preparations (dilutions 1:1000 for anti-I(Ma)serum, and, 1:100 for anti-SSEA-1 ascites or ^{125}I-labelled R.communis (60 ng containing 1 x 10^5 cpm in 1% BSA in PBS) were added and the plates incubated at $4^{\circ}C$ for 16 hours. The plates were then washed five times with PBS.

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For the antibody assays, ^{125}I-labelled rabbit
antibodies to human [for the anti-I (Ma) antibody] and mouse
(for anti-SSEA-1) immunoglobulins were added (50 microlitres
containing 10^5 cpm and 30 ng of protein per well).

For the radioassay using ^{125}I labelled lectin, the
plates were dried and the wells counted using a Nuclear
Enterprises 1600 counter.

Inhibition assay procedures were adapted from Wood,
E., et.al. (1979) Mol. Immunol. 16, 813-819; and Gooi, H.C.,
et.al; (1985) Mol. Immunol. 22, 689-693. using serial
dilutions of the neoglycolipids incorporated into liposomes
as inhibitors of the binding of the anti-I(Ma) $(1:10^4$
dilution) or R.communis lectin (30 ng containing 5 x 10^4
cpm) to sheep gastric mucins or meconium glycoproteins of
the non-secretor type (500 ng per well).   The composition
of the liposomes was neoglycolipid, lecithin (dimiristoyl
phosphatidyl choline) and cholesterol.   Further details are
discussed in Tang P.W. et al Biochem Biophys Res Comm Vol.
132, pages 474-480 (1985)

Results and Discussion

(i)   The anti-I(Ma) antibody bound to oligosaccharides
O$_4$, O$_1$ and N$_8$ which all contain the saccharide
sequence Gal-beta-1-4GlcNAc-beta-1-6- .   Reference is made
to Fig.2A which shows the reactivity of anti-I(Ma) to the
neoglycolipids on PVC plates.   Positive binding was
observed with as little as 10 to 50 ng (10 to 100 picomoles)
of the oligosaccharides but no binding was detected with
normal human serum or with a negative control of conjugate
containing PPEADP linked to lactose.

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(ii) It has previously been reported that the carbohydrate
chains of sheep gastric mucins express I(Ma) as well as
other antigenic determinants and that there is a 10,000-fold
reduction of the antigenicity of the oligosaccharides after
release from the bound peptide moiety [Wood, E., et.al.,
(1981) Carbohydr. Res., 90, 269-282].   Fig. 2B shows the
results for inhibition-of-binding assay of anti-I(Ma) to
sheep gastric mucins on PVC plates.   The data indicate that
when oligosaccharides derived from sheep gastric mucins are
incorporated into a conjugate of this invention, using
PPEADP and incorporated into liposomes, the antigenic
activity of the oligosaccharide in inhibition assays is
virtually indistinguishable from that of the original
untreated mucin glycoprotein .

(iii) The oligosaccharide O$_4$, containing the I(Ma) antigen
sequence produced a radioactive band on HP-TLC with
immunostaining as described above, with as little as three
micrograms of oligosaccharide.   No immunostaining was
detectable for lactose.   Fig. 2C shows the results of
binding of anti-I(Ma) to neoglycolipids on a TLC plate.

(iv) In PVC wells coated with the neoglycolipid obtained by
reductive amination of LNFP III alditol which contains the
carbohydrate sequence Gal-beta-1-4(Fuc-alpha-1-3)GlcNAc of
the stage specific embryonic antigen of mouse, SSEA-1, the
oligosaccharide specifically bound the hybridoma antibody,
anti-SSEA-1, (Fig.2D).   The chemically synthesised
trisaccharide analogue 3F, after reduction to the 3F alditol
and

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conjugation to PPEADP, gave no binding above the control
mouse serum even at the highest level (500 ng) tested.
The difference may be due to the loss of one carbon unit
from the N-acetylglucosamine residue of the 3F alditol
during periodate oxidation, since the conjugate containing
3F without prior reduction gave substantial binding, or to a
difference in orientation of the oligosaccharide moiety.

(v)   Conjugates containing reduced oligosaccharide O_4 and
N-acetyllactosamine were coated on to PVC plates, and shown
to bind the ^{125}I- labelled lectin R. communis which is
known to react strongly with the sequence Gal-beta-1-4GlcNAc
(Fig.2E).   A conjugate containing maltose and PPEADP gave
no binding, nor did PPEADP itself.

(vi) The conjugates containing the oligosaccharide O_4 and
N-acetyllactosamine were incorporated into liposomes and
were shown to inhibit specifically binding of the lectin to
human meconium glycoproteins (Fig 2F).

EXAMPLE 3

     Reduced O-linked and reducing N-linked oligosaccharide
chains were obtained from galactosyltransferase, an enzyme
from human milk, by alkaline borohydride degradation and
hydrazinolysis and purified by ion-exchange
chromatography.   Three preparations of the enzyme were
used: (1) combined galactosyltransferase from donors of
unknown blood group; (2) combined milk from blood group A
donors; and, (3) milk from a single donor of blood group O
(non-secretor).   For deglycosylation an aliquot of
galactosyltransferase

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preparation 3 was treated with anhydrous hydrogen fluoride
for one hour at 4°C.   The mixture was extensively
dialysed against water then lyophilised and the residue
redissolved in isotonic saline.

Galactosyltransferase (600 micrograms of preparations
1 and 3 or 300 micrograms of preparation 2) containing 5% of
hexose as determined by phenol sulphuric acid assay using
preparation 3, was treated with 0.05M NaOH/M $NaBH_4$ (200
microlitres) at 50°C for 24 hours to release the O-linked
oligosaccharides.   After acidification with acetic acid to
pH 5.0 the mixture (250 microlitres) was applied to a column
(0.4 x 1cm) of Dowex AG50W-X8 (Trade Mark) (H^{+}) resin
(200-400 mesh).   Elution with water (1.2 ml) gave the
O-linked oligosaccharide fraction.   The fraction was
evaporated to dryness at 60°C _in vacuo_ and methanol (200
microlitres) was thrice evaporated from the residue to
remove boric acid.

Elution with 3M ammonium hydroxide (1.2 ml) yielded
the peptides and glycopeptides with the N-linked
oligosaccharides.   This fraction was evaporated to dryness
at 60°C _in vacuo_ and water (500 microlitres) was
evaporated from the residue which was then subjected to
hydazinolysis and N-acetylation.   The products (250
microlitres) were chromatographed on the same resin as
described above.   The unretained fraction contained the
N-linked oligosaccharides.   Fractions corresponding to the
O- and N-linked oligosaccharides were also obtained from
preparation 3 which had been deglycosylated as described
above.

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The oligosaccharides were resolved by HP-TLC. on
silica gel 60 (Merck 5641, Trade Mark) using butan-1-ol:
acetone:water, 6:5:4, and visualised with 0.2% orcinol in 2M
ethanolic sulphuric acid.

For antibody binding assays on PVC plates and
inhibition-of-binding assays, reduced O-linked fractions
released from galactosyltransferase (100-600 micrograms)
were dried at 60°C in vacuo and oxidised with sodium
periodate (10-60 micrograms as appropriate) in 40mM
imidazole hydrochloride (pH 6.5, 100 microlitres) at 0°C
for 45 minutes.   To the resulting mixtures, chloroform/
methanol (1:1) was added (240-480 microlitres as
appropriate) containing PPEADP (240-480 micrograms) and
NaCNBH$_3$ (100-200 micrograms) and the mixtures incubated at
50°C for 16 hours.   The reducing oligosaccaharides
released by hydrazinolysis of galactosyltransferase (100 to
600 micrograms) were coupled to PPEADP without periodate
oxidation.   The entire reaction mixtures were used for
antibody binding on PVC plates and inhibition-of-binding
assays.

For chromatogram binding assays, O-linked
oligosaccharides from 15 micrograms of galactosyltransferase
(preparation 1) were subjected to HP-TLC, oxidised with
periodate in situ and coupled to PPEADP as described in
Example 2.   N-linked oligosaccharides from 15 micrograms of
galactosytransferase were chromatographed and coupled to
PPEADP without periodate oxidation.

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EXAMPLE 4

Immunochemical and chromatographic studies were carried out, using the procedures described in Example 2, on keratan sulphate proteoglycans which express antigenic determinants recognised by several monoclonal antibodies.

Ten sulphated oligosaccharides were purified from bovine corneal keratan sulphate after end-beta-galactosidase digestion according to the procedure described in Scudder et al, Eur. J. Biochem. 157, 365-373 (1986).

The oligosaccharides were converted to neoglycolipids with PPEADP.   For chromatograhic studies, the conversion was carried out in situ.

When bound to PVC plates or to the chromatograhic support, it was possible to effect a variety of immunological tests on the oligosaccharides which in this immobilised form were presented in spatial configuration which permitted antibody binding.

The results of this work are reported in Eur. J. Biochem. (1986) 157, 385-391
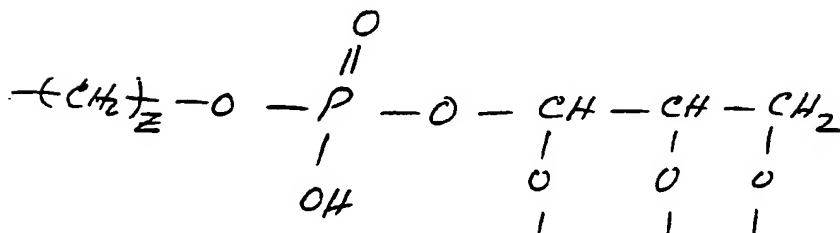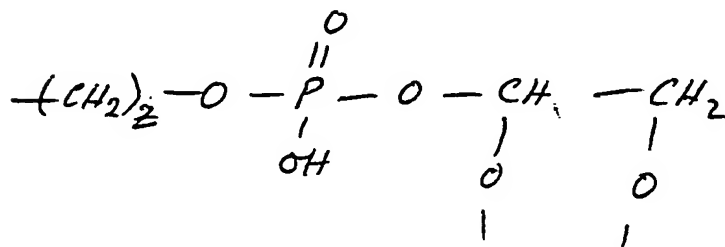
-36-

CLAIMS

1.   A biochemical reagent comprising a conjugate of an
      oligosaccharide and an immobilising carrier, said
      oligosaccharide being bound in spaced relationship to
      the carrier by means of an interposed spacer molecule
      whereby the oligosaccharide is presented in
      antigenically active steric configuration for binding
      on encountering an antibody.

2.   A reagent as claimed in claim 1, in which the spacer
      molecule is a lipid and the combination thereof with
      the oligosaccharide is a neoglycolipid.

3.   A reagent as claimed in claim 2, in which the lipid
      has at least two hydrophobic chains.

4.   A reagent as claimed in claim 3, in which the
      neoglycolipid has the general formula:

$$R^1 \text{------} NH - X \begin{smallmatrix} \diagup R^2 \\[2pt] \diagdown R^4 \end{smallmatrix} - R^3$$
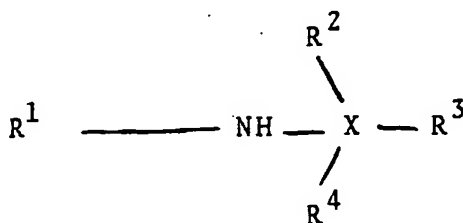
-37-

where R^1 represents a carbohydrate group, R^2
represents an alkyl, alkoxy or alkoyl group, R^3
represents an alkyl, alkoxy or alkoyl group, R^4
represents an alkyl, alkoxy or alkoyl group, or
hydrogen, R^2, R^3 and R^4 being the same or
different groups, z is zero or 1,2, or 3 and X
represents a linking group selected from:

$$-(CH_2)_z-O-\overset{\overset{\displaystyle O}{\parallel}}{\underset{OH}{P}}-O-\underset{\underset{|}{O}}{CH}-\underset{\underset{|}{O}}{CH_2}$$

$$-(CH_2)_z-O-\overset{\overset{\displaystyle O}{\parallel}}{\underset{OH}{P}}-O-\underset{\underset{|}{O}}{CH}-\underset{\underset{|}{O}}{CH}-\underset{\underset{|}{O}}{CH_2}$$

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5.   A reagent as claimed in claim 2 or 3 or 4, in which
      the group X is a residue of a compound selected from
      phosphatidyl ethanolamine dipalmitoyl (PPEADP),
      L-alpha- phosphatidyl-L-serine, L-alpha-phosphatidyl-
      ethanolamine (PPEA) dilauroyl, PPEA dimyristoyl, PPEA
      distearoyl, PPEA beta-oleoyl-gamma-palmitoyl.

6.   A reagent as claimed in any preceding claim, in which
      the oligosaccharide is derived from an glycoprotein or
      proteoglycan.

7.   A reagent as claimed in any preceding claim, in which
      the carrier is selected from a chromatographic support
      material, a plate of plastics material and a liposome.

8.   A reagent as claimed in claim 7, in which the plastics
      material is polyvinyl chloride.

9.   A reagent as. claimed in claim 7, in which the
      chromatographic support is silica gel.

10.   A method of preparing a biochemical reagent,
      comprising reacting a reducing oligosaccharide and an
      amine derivative of a spacer compound to produce a
      neoglycolipid and bonding the reaction product to an
      immobilising carrier.

11.   A method of preparing a biochemical reagent as claimed
      in claim 10, in which the oligosaccharide is liberated
      from an immunogenic glycoprotein or proteoglycan.

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12. A method of preparing a biochemical reagent as claimed
    in claim 11, in which the oligosaccharide is liberated
    by enzyme action.

13. A method of preparing a biochemical reagent as claimed
    in claim 11, in which the oligosaccharide is liberated
    by the action of alkaline borohydride, followed by
    oxidation with periodate to form an aldehyde group.

14. A method of preparing a biochemical reagent as claimed
    in claim 11, in which the oligosaccharide is liberated
    by the action of hydrazine, followed by acylation and
    oxidation with periodate to form an aldehyde group.

15. A method of preparing a biochemical reagent as claimed
    in any of claims 10 to 14, in which the amine and the
    oligosaccharide are reacted in the presence of
    cyanoborohydride to form a neoglycolipid.

16. A method of conducting a biochemical assay, comprising
    providing a biochemical reagent comprising a conjugate
    of an oligosaccharide and an immobilising carrier,
    said oligosaccharide being bound in spaced
    relationship to the carrier by means of an interposed
    spacer molecule whereby the oligosaccharide is
    presented in antigenically active steric configuration
    for binding on encountering an

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antibody, contacting said conjugate with an antibody
to the oligosaccharide whereby binding of the antibody
and the oligosaccharide moiety of the conjugate occurs
and detecting that with an analytically detectable
reagent.

17.   A method of conducting an immunoassay as claimed in
      claim 16, including the step of treating the
      conjugate-bound antibody with a second conjugate of an
      oligosaccharide with a liposome carrier.

18.   A method of conducting an immunoassay as claimed in
      claim 16, in which the analytically detectable reagent
      is a radiolabelled antibody to the bound antibody and
      detection thereof is effected by autoradiography.

19.   A biochemical assay kit comprising a test plate having
      thereon an immobilised oligosaccharide, as claimed in
      any of claims 1 to 9, a reagent comprising a solution
      of a neoglycolipid of which the glyco group is the
      said oligosaccharide and a further reagent comprising
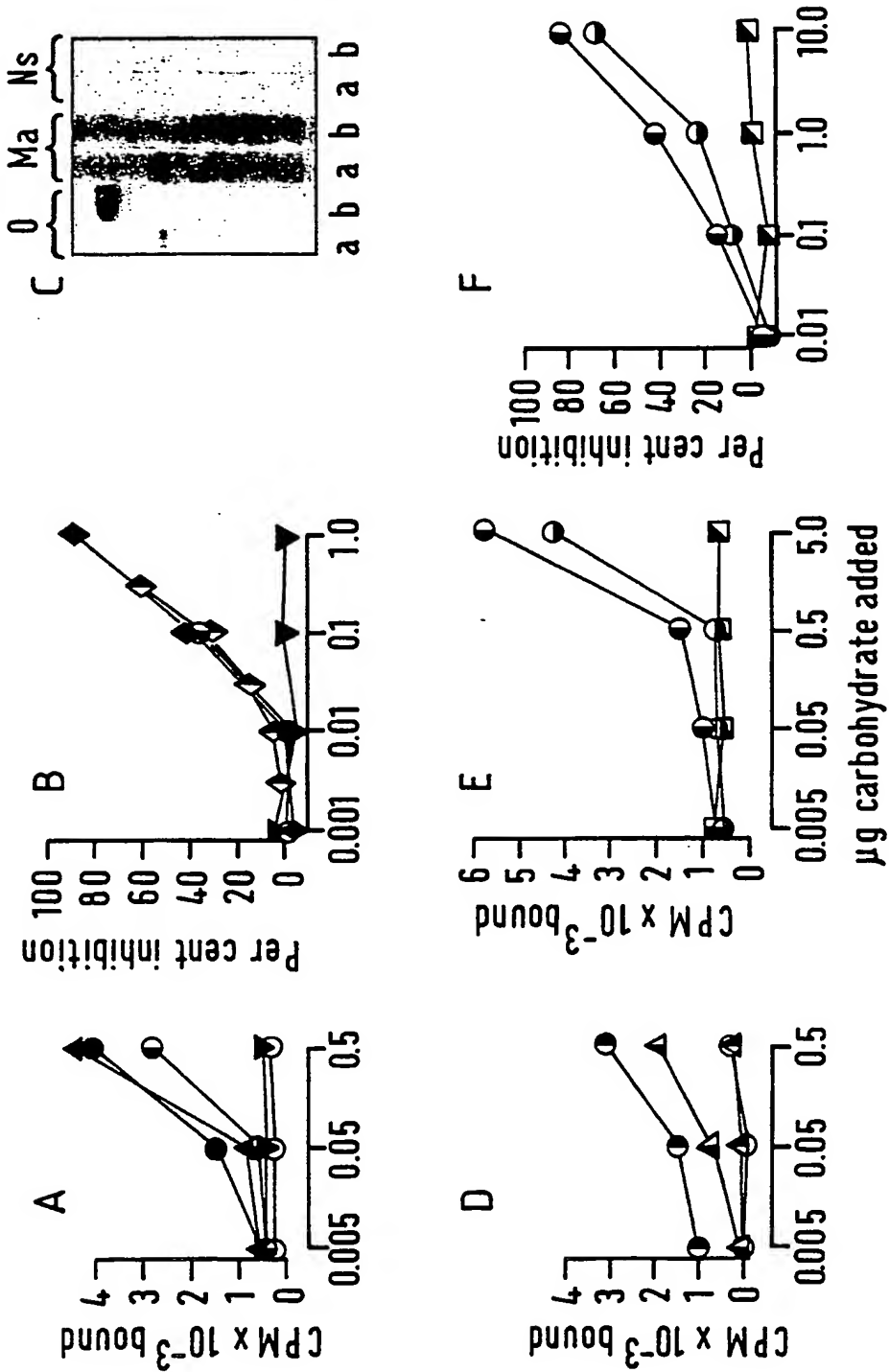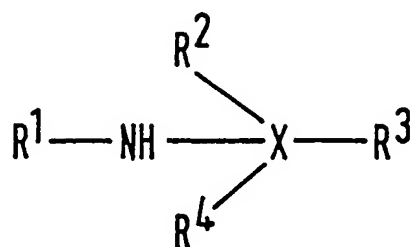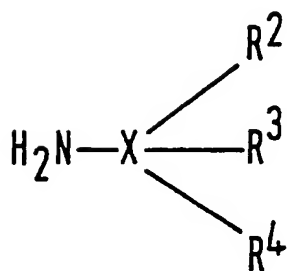   a solution of liposome-forming components.
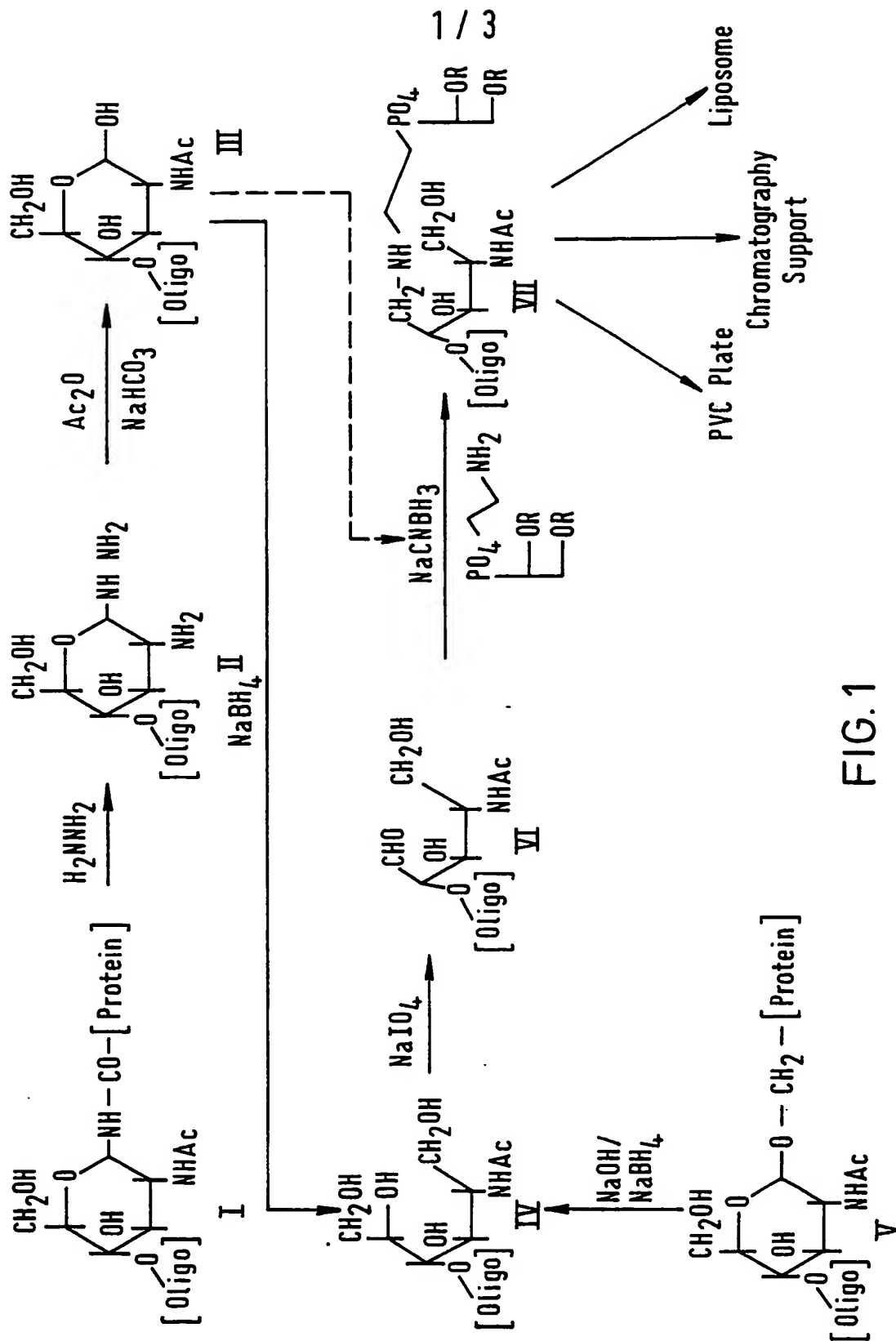
FIG.1
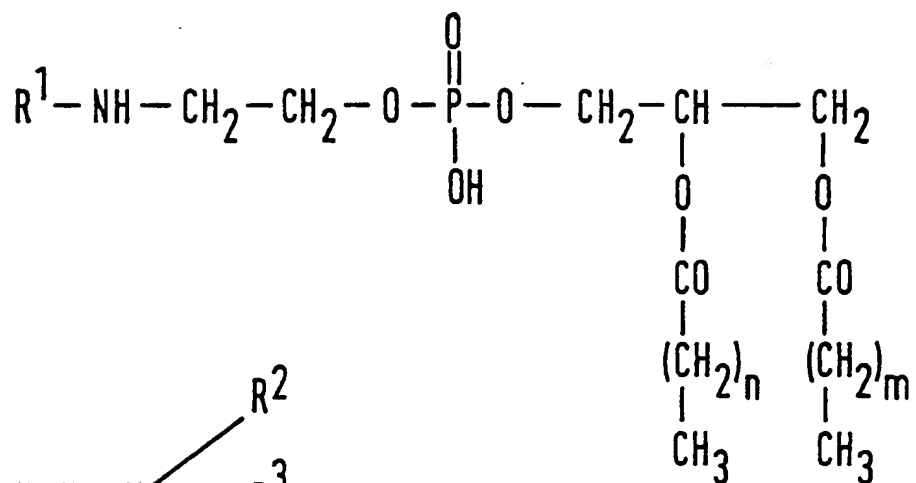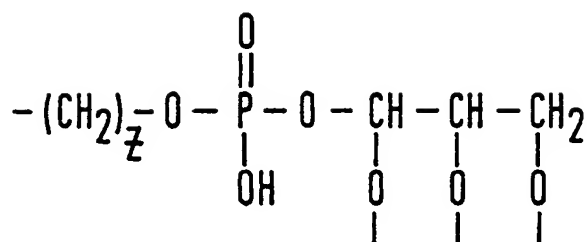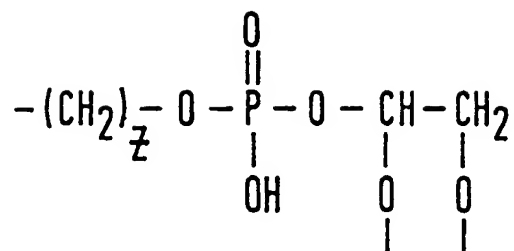
FIG. 2

$$-(CH_2)_{\overline{z}}-O-\overset{\overset{\displaystyle O}{\|}}{\underset{\underset{\displaystyle OH}{|}}{P}}-O-\overset{\underset{\underset{\displaystyle |}{\displaystyle O}}{|}}{CH}-\overset{\underset{\underset{\displaystyle |}{\displaystyle O}}{|}}{CH_2}$$

$$-(CH_2)_{\overline{z}}-O-\overset{\overset{\displaystyle O}{\|}}{\underset{\underset{\displaystyle OH}{|}}{P}}-O-\overset{\underset{\underset{\displaystyle |}{\displaystyle O}}{|}}{CH}-\overset{\underset{\underset{\displaystyle |}{\displaystyle O}}{|}}{CH}-\overset{\underset{\underset{\displaystyle |}{\displaystyle O}}{|}}{CH_2}$$

$$R^1-NH-CH_2-CH_2-O-\overset{\overset{\displaystyle O}{\|}}{\underset{\underset{\displaystyle OH}{|}}{P}}-O-CH_2-CH-CH_2$$

with branches:

$$\begin{array}{cc} CH & CH_2 \\ | & | \\ O & O \\ | & | \\ CO & CO \\ | & | \\ (CH_2)_n & (CH_2)_m \\ | & | \\ CH_3 & CH_3 \end{array}$$

$$H_2N-X\begin{cases} R^2 \\ R^3 \\ R^4 \end{cases}$$

$$R^1-NH-X\begin{array}{c} R^2 \\ -R^3 \\ R^4 \end{array}$$

INTERNATIONAL SEARCH REPORT

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) °

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC⁴: G 01 N 33/531; G 01 N 33/547

II. FIELDS SEARCHED

Minimum Documentation Searched ⁷

Classification System	Classification Symbols
IPC⁴	G 01 N 33; C 07 H

Documentation Searched other than Minimum Documentation
to the Extent that such Documents are included in the Fields Searched ⁸

III. DOCUMENTS CONSIDERED TO BE RELEVANT °

Category °	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³
X	Chemical Abstracts, volume 104, no. 13, 31 March 1986, Columbus, Ohio (US) P.W. Tang et al.: "Novel approach to the study of the antigenicities and receptor functions of carbohydrate chains of glycoproteins", see page 542, abstract 107512m, & Biochem.Biophys. Res.Commun. 1985, 132(2)474-80 (Eng). --	1-16
X	Chemical Abstracts, volume 95, no. 23, 7 December 1981, Columbus, Ohio (US) C.Wood et al.: "Immunochemical studies of conjugates of isomaltosyl oligo-saccharides to lipid. I. Antigenicity of the glycolipids and the production of specific antibodies in rabbits see page 496, abstract 201810z, & J. Exp.Med. 1981, 154(2), 432-49(Eng). --	10,15

° Special categories of cited documents: ¹⁰

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
22nd January 1987	2 0 FEV. 1987
International Searching Authority	Signature of Authorized Officer
EUROPEAN PATENT OFFICE	M. VAN MOL

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category*	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
X	Chemical Abstracts, volume 94, no. 21, 25 May 1981, Columbus, Ohio (US) E. Wood et al.: "Preparative, affinity chromatography of sheep gastricmucins having blood-group Ii activity, and release of antigenically active oligosaccharides by alkaline-borohydride degradation", see page 529, abstract 172612h & Carbohydr.Res.1981, 90(2) 269-82 (Eng).	11,13
X	Chemical Abstracts, volume 103, no. 3, 22 July 1985, Columbus, Ohio (US) P.W. Tang et al.: "An improved method for the liquid chromatography of the 1-deoxy-1-(2-pyridylamino)alditol derivatives of oligosaccharides and its application to structural studies of the carbohydrate moieties of glycoproteins", see abstract 19247c, & Carbohydr. Res. 1985, 136, 259-71 (Eng.)	11,14
A	Chemical Abstracts, volume 96, no. 1, 4 January 1982, Columbus, Ohio (US) C. Wood et al.: "Immunochemical studies of conjugates of iso-maltosyl oligosaccharides to lipid: specificities and reactivities of the antibodies formed in rabbits to stearyl-isomaltosyl oligosaccharides" see page 443, abstract 4762e, & Arch.Biochem.Biophys. 1981, 212(1) 262-76(Eng).	1-5
A	Chemical Abstracts, volume 96, no. 1, 4 January 1982, Columbus, Ohio (US) C. Wood et al.: "Immunochemical studies of conjugates of isomaltosyl oligosaccharides to lipid: fractionation of rabbit antibodies to stearyl-iso-maltosyl oligosaccharides and a study of their combining sites by a competitive binding assay", see page 443, abstract 4763f, & Arch.Biochem.Biophys. 1981, 212(1) 277-89 (Eng).	1-5
A	EP, A, 0144084 (K.K. TOSHIBA) 12 June 1985, see page 14, examples 10-23	5

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON

INTERNATIONAL APPLICATION NO.   PCT/GB 86/00659 (SA   15033)
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This Annex lists the patent family members relating to the
patent documents cited in the above-mentioned international
search report. The members are as contained in the European
Patent Office EDP file on 11/02/87

The European Patent Office is in no way liable for these
particulars which are merely given for the purpose of
information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A- 0144084	12/06/85	JP-A- 60117159	24/06/85